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Cephalopod alcohol dehydrogenase: purification and enzymatic characterization

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Octopus, squid and cuttle-fish organs were examined for alcohol dehydrogenase activity. Only one form was detectable, with properties typical of mammalian class III alcohol dehydrogenase. The corresponding protein was purified from octopus and enzymatically characterized. Ion-exchange and affinity chromatography produced a pure protein in excellent yield (73%) after 1600-fold purification. Enzymatic parameters with several substrates were similar to those for the human class III alcohol dehydrogenase, demonstrating a largely conserved function of the enzyme through wide lines of divergence covering vertebrates, cephalopods and bacteria. The results establish the universal occurrence of class III alcohol dehydrogenase and its strictly conserved functional properties in separate living forms. The absence of other alcohol dehydrogenases in cephalopods is compatible with the emergence of the ethanol-active class I type at a later stage, in lineages leading to vertebrates.

Cephalopod enzyme; Protein evolution; Alcohol dehydrogenase; Glutathione-dependent formaldehyde dehydrogenase; Octopus protein; Enzymatic properties

1. INTRODUCTION

Two different classes of human alcohol dehydrogenase, classes I and III with separate substrate specificities, have a related origin through an ancestral duplication initially time-estimated by analysis of an amphibian enzyme [1]. The duplication was further ascertained by analysis of lower vertebrate forms (cf. [2]), and finally established by detection of molecules with hybrid properties in piscine lines, allowing the tracing of emerging enzyme activity [3]. Still, however, the exact dating of the duplicatory event is uncertain, and analysis of the enzyme from further animal forms originating early in the evolutionary line is essential.

We have therefore now studied alcohol dehydrogenase activity in cephalopod species and purified the only form of the enzyme detectable in *Octopus vulgaris*, constituting the first characterized cephalopod form of this enzyme. It behaves like a mammalian class III alcohol dehydrogenase, with very low affinity for ethanol, and a high glutathione-dependent formaldehyde dehydrogenase activity. Overall, these results establish the early origin of the class III type of alcohol dehydrogenase, compatible with its constant [4] evolutionary pattern, and suggests a fundamental importance of this enzyme in basic cellular functions.

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2. MATERIALS AND METHODS

Organs from three cephalopod species were examined, Western Mediterranean octopus (*Octopus vulgaris*), Mediterranean squid (*Loligo vulgaris*) and Mediterranean cuttle-fish (*Sepia officinalis*). Hepatopancreas, stomach, caecum, gills, salivary glands, gonads, eye, branquial heart, skin, oesophagus and muscle were removed in separate batches by dissection, cleaned and washed in ice-cold distilled water, and stored at −20°C.

For activity measurements, organs were cut in small pieces, homogenized with a Polytron instrument (Kinemática GmbH, Luzerne, Switzerland) in 10 mM Tris-HCl/0.5 mM dithiothreitol, pH 8.5, and centrifuged at 27,000 × *g* for 30 min at 4°C. Enzyme activity was determined spectrophotometrically at 25°C by monitoring the change in absorbance at 340 nm with a Cary 219, a Hitachi 220s, or a Hewlett Packard 8452A diode array spectrophotometer. Alcohol oxidation was measured with 4 mM NAD^+ in 0.1 M glycine/NaOH, pH 10, while aldehyde reduction was measured with 1.28 mM NADH in 0.1 M sodium phosphate, pH 7.5. Formaldehyde dehydrogenase activity was measured in 0.1 M sodium phosphate, pH 8.0, with *S*-hydroxymethylglutathione (formed by spontaneous reaction of formaldehyde and glutathione) as substrate [5]. The substrate used during purification was 1 mM octanol at pH 10.0. One unit of activity (U) corresponds to 1 μmol NAD(H) formed per minute.

For enzyme purification from *Octopus vulgaris*, gills, salivary glands and the branquial heart were dissected, homogenized, and centrifuged as above. The supernatant was then ultracentrifuged at 85,000 × *g* for 1 h at 4°C. After dialysis against 3 × 2 liters changes of 10 mM Tris-HCl/0.5 mM dithiothreitol, pH 8.5, the material was applied to DEAE-Sepharose CL-6B (2.5 × 28 cm) in the same buffer. After washing at 45 ml/h for 700 ml, a linear gradient of increasing NaCl (0–150 mM in 500 ml) eluted the enzyme activity. Active fractions were concentrated to 20 ml (Amicon concentrator with a Diaflo PM 10 membrane), dialyzed (3 × 2 liters) against 10 mM Tris-HCl/0.5 mM dithiothreitol, pH 7.9, and applied to Blue-Sepharose (1 × 25 cm). After isocratic elution at 20 ml/h (100 ml) a concentration gradient of Tris-HCl (10–100 mM in 50 ml) in 0.5 mM dithiothreitol, pH 7.9, was applied, followed by 100 mM Tris-HCl/0.5 mM dithiothreitol (90 ml),

and a gradient (0–1.7 mM in 500 ml) of NADH in the same buffer. When octanol dehydrogenase emerged, a pulse of 1.7 mM NADH was applied. Active fractions were pooled, concentrated to 1 ml and dialysed against 0.1 M Tris-HCl/0.5 mM dithiothreitol, pH 7.9, for removal of NADH and stored at −80°C.

Starch gel electrophoresis under native conditions was performed with both alcohol and formaldehyde dehydrogenase staining [6,7]. Electrophoresis in SDS/polyacrylamide gels [8] utilized subsequent silver staining [9], and isoelectric focusing was performed in a vertical polyacrylamide minigel system [10].

3. RESULTS

3.1. *Species and organ distribution*

Three cephalopod species were examined, octopus (*Octopus vulgaris*), squid (*Loligo vulgaris*) and cuttle-fish (*Sepia oficinalis*). In each case, hepatopancreas, stomach, caecum, gills, salivary glands, gonads, eye, branquial heart, skin, oesophagus, and muscle, were examined. Direct activity measurements of organ homogenates at pH 10.0 with 5 mM ethanol, 33 mM ethanol, 0.5 M ethanol, 1 mM octanol, and 100 mM 2-buten-1-ol (crotyl alcohol) revealed that all three species have similar tissue distributions of alcohol dehydrogenase and that all organs exhibited high K_m activities, but no low K_m activities. Homogenates from stomach and caecum were also tested with 1 mM and 50 mM isopropanol in order to detect, if present, the short-chain type of alcohol dehydrogenase typical of *Drosophila*, but no activity was detectable under these circumstances.

Starch gel electrophoresis of homogenates under native conditions and subsequent activity staining with 33 mM ethanol or 0.1 M ethanol and 0.1 M crotyl alcohol revealed only one band of activity (or group of bands upon overloading or sample aging; cf. below) detectable with crotyl alcohol and present in most organs, but different in each species (Fig. 1), and no band with ethanol in any of the organs analyzed. The same band appeared with staining for glutathione-dependent formaldehyde dehydrogenase, indicating that this band corresponds to class III alcohol dehydrogenase. In conclusion, therefore, the results demonstrate that all cephalopods tested have a single form of alcohol dehydrogenase, which is of fairly wide tissue distribution and has overall properties typical of class III alcohol dehydrogenase, but that cephalopods do not contain appreciable amounts of any other forms of alcohol dehydrogenase.

3.2. *Purification of octopus alcohol dehydrogenase*

Activity in octopus was in general higher than in the other species and was therefore used for purification of the alcohol dehydrogenase detected above. The most active organs, gills, salivary glands and branchial heart (170 g), were homogenized, centrifuged (27,000 × *g* for 30 min at 4°C), and ultracentrifuged (85,000 × *g* for 1 h at 4°C). Muscle was also very active but could not be used because of difficulty in homogenization. After dialysis against 10 mM Tris-HCl/0.5 mM dithiothreitol, pH 8.5, the material was submitted to DEAE-Sepharose Cl-6B chromatography utilizing a gradient of increasing NaCl concentration as given in the methods sections. The active fractions were pooled, concentrated, changed to 10 mM Tris/HCl, 0.5 mM dithiothreitol, pH 7.9, and loaded to Blue-Sepharose. Elution was achieved with first an increasing concentration of Tris-HCl, then a NADH gradient, and when alcohol dehydrogenase activity with 1 mM octanol was appearing, elution with a pulse of 1.7 mM NADH. Active fractions were pooled, concentrated to 1 ml, dialyzed against 0.1 M Tris-HCl, 0.5 mM dithiothreitol, pH 7.9, and stored at −80°C. Final yield and purification showed excellent values, 73% yield at a purification of 1600-fold (Table I). The material obtained corresponds to the enzyme detectable upon direct activity measurements in intact homogenates (Fig. 1). It gives a group of closely migrating bands upon starch gel electrophoresis and subsequent activity staining under native conditions. A single

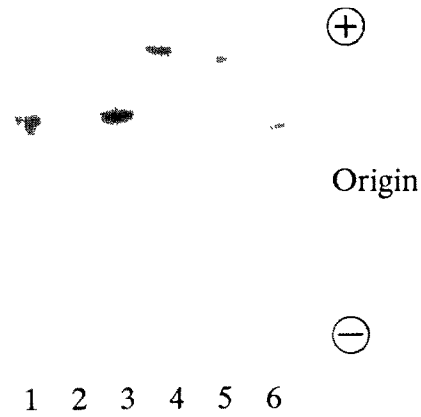

Fig. 1. Starch gel electrophoresis of homogenates from several organs of cephalopods. Activity staining using crotyl alcohol as substrate. Octopus: branquial heart (lane 1), skin (2) and muscle (3). Cuttle-fish: muscle (4) and eye (5). Squid muscle (6).

Table I

Purification of octopus alcohol dehydrogenase

Purification step	Protein (mg)	Activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Supernatant (crude extract)	2150	5.2	0.002	1	100
DEAE-Sepharose	21	4.4	0.2	100	84
Blue-Sepharose	1.1	3.8	3.3	1600	73

Gills, branquial heart and salivary glands (170 g), were used. Activity was measured with 1 mM octanol and 4 mM NAD^+ in 0.1 M glycine–NaOH, pH 10.0.

Fig. 2. SDS/polyacrylamide gel electrophoresis of the purified enzyme. Lane 1, molecular weight standards (66, 45, 40, 29 and 20.1 kDa); lane 2, purified enzyme.

band is detected upon SDS/polyacrylamide electrophoresis under denatured conditions, with a subunit molecular weight corresponding to about 40,000 (Fig. 2). Several bands (pI range 6.8–7.0) were detected by isoelectric focusing, as noticed also for class III alcohol dehydrogenase from several other species [3,11].

3.3. *Enzymatic characterization*

The purified enzyme was tested for activity with aliphatic alcohols and ω-hydroxyfatty acids. With ethanol, it was active only at very high concentrations and could not be saturated with this substrate, nor with pentanol (Table II). Tests with NAD and NADP demonstrated a clear NAD preference, with a 43-fold ratio between the NAD/NADP activities at pH 10. The enzyme exhibited low activity with different aldehydes (hexanal, *trans*-2-hexenal, and octanal) and NADH. However, the enzyme was highly active with formaldehyde in the presence of glutathione, under these conditions also demonstrating a clear utilization of NAD^+ (Table II). All these properties are typical for the class III type of human alcohol dehydrogenase. Thus, human tissues [12], octopus (this work), intermediately positioned species [1,3], and *Escherichia coli* [13], all contain fairly identical enzyme activities of class III type, exhibiting activity toward long-chain alcohols in the absence of glutathione and high specificity for formaldehyde in the presence of glutathione, but non-saturable and extremely low activity toward ethanol under both conditions (Table III).

Mammalian class III alcohol dehydrogenase can be activated with hydrophobic anions [15] and this property is known to be correlated with the presence of Arg-115 [16]. We therefore tested the possibility to activate octopus alcohol dehydrogenase with hydrophobic anions, by activity measurements in the absence and presence of 2 mM octanoate. The results indicate a clear activation, over 3-fold regarding ethanol oxidation at both pH 7.5 and 10, and 5-fold regarding acetaldehyde reduction at pH 7.5 (Fig. 3). Similarly, while octanoate enhances the oxidation of ethanol, it inhibits that of octanol, with a K_i of about 2 mM at pH 10.

Table II

Enzymatic properties of octopus alcohol dehydrogenase

Substrate	K_m	k_{cat}	k_{cat}/K_m
Ethanol	NS	–	0.086
Pentanol	NS	–	6
Octanol	1.45 (2.8)	710 (13)	490 (5)
10-OH-decanoate	0 19 (0.17)	730 (110)	3,800 (650)
12-OH-dodecanoate	0.29	770	2700
16-OH-hexadecanoate	0.16	40	250
S-Hydroxymethylglutathione	0.0015	300	200,000
NAD^+ at 0.02 mM HMG	0.035	–	–

Values measured at pH 10 (pH 7.5 within parentheses). NS, non-saturable. Glutathione-dependent formaldehyde dehydrogenase activity with S-hydroxymethylglutathione (HMG) was measured at pH 8.0.

4. DISCUSSION

The results demonstrate that alcohol dehydrogenase of class III type, i.e. the glutathione-dependent formaldehyde dehydrogenase, is present in cephalopod species in general. It has a wide tissue distribution, but with above-average occurrence in gills, salivary glands, branquial heart, caecum, muscle and skin. Activity staining after electrophoresis under native conditions demonstrated only one enzyme form and with similar activity relationships in all cases. However, this single form of the enzyme has slightly different physicochemical properties, dependent on the species, and the electrophoretic migration under native conditions differs between octopus, squid, and cuttle-fish. The finding of only one alcohol dehydrogenase, a class III enzyme, further demon-

Table III

Comparison of enzymatic properties of class III alcohol dehydrogenase from highly divergent species

Substrate	k_{cat}/K_m ($mM^{-1} \cdot min^{-1}$)				
	Human	Rat	Frog	Octopus	*E. coli*
Ethanol	0.045	ND	ND	0.086	0.084
Octanol	190	280	560	490	0 27
12-Hydroxydodecanoate	2,800	2,300	3,100	2,700	630
S-Hydroxymethylglutathione	50,000	235,000	149,000	200,000	99,000

Activity was determined at pH 10.0 for alcohols and at pH 8.0 with *S*-hydroxymethylglutathione. The octopus data are from this study, those from the human and *E coli* enzymes from [13], the rat enzyme form [5,14], and the frog enzyme form [1].

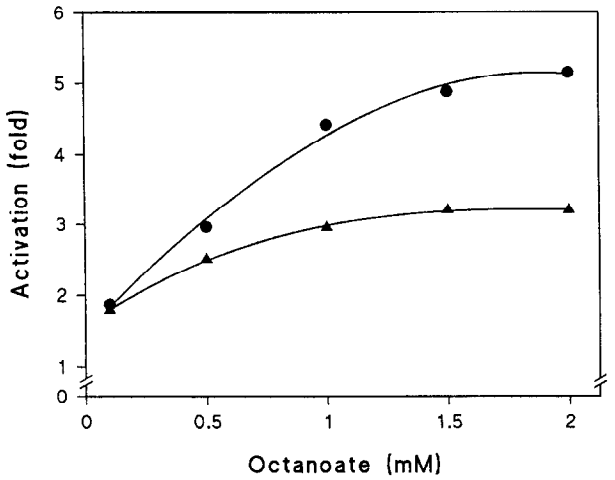

Fig 3. Activation of octopus alcohol dehydrogenase with octanoate upon ethanol oxidation (●) and acetaldehyde reduction (▲). Activities were measured with 0.5 M ethanol in 0.1 M glycine/NaOH, pH 10.0, and with 0.1 M acetaldehyde in 0.1 M sodium phosphate, pH 7.5.

strates that normal multicellular organisms manage well without ethanol active alcohol dehydrogenase forms. Lack of class I alcohol dehydrogenase has been noticed before in a strain of deer-mouse [17], but that animal still contains an ethanol metabolizing enzyme in gastric mucosa [18,19], probably a class IV alcohol dehydrogenase. The cephalopods, therefore, are the first multicellular organisms known without appreciable amounts of any alcohol dehydrogenase capable of metabolizing low molecular weight alcohols.

The enzyme was purified from octopus and obtained in excellent yield after a 1600-fold purification. This result demonstrates a fairly stable enzyme, present in low abundance (0.002 U/mg protein in the organs with most activity). Enzymatic characterization showed it to be a typical glutathione-dependent formaldehyde dehydrogenase, with considerable activity toward long-chain alcohols in the absence of glutathione, but nonsaturable with ethanol and consequently with extremely low activity toward that substrate. Coenzyme specificity showed a clear preference for NAD^+. Overall properties of the octopus protein are almost identical to those for bacterial, amphibian, mammalian and human alcohol dehydrogenase class III forms (Table III).

We conclude that cephalopod species in general contain the class III type of alcohol dehydrogenase with a wide tissue distribution and that the enzyme is closely similar to the corresponding forms in highly divergent organisms, demonstrating that class III is functionally strictly conserved over wide ranges (Table III). No class I type or other class of alcohol dehydrogenase was detectable in any of the cephalopod species or organs tested. Combined, all results emphasize the basic importance of class III alcohol dehydrogenase and show its early evolutionary origin, apparently present in conserved form in the absence of the class I type. This confirms suggestions from analysis of just the vertebrate enzymes [1,2], and supports the view that the class I/III duplication occurred after the divergence of vertebrates from early evolutionary lines.

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